AD_____

Award Number: DAMD17-99-1-9280

TITLE: 99m Tc-peptides for Detection of Breast Cancer

PRINCIPAL INVESTIGATOR: Mary Rusckowski, Ph.D.

CONTRACTING ORGANIZATION: University of Massachusetts
Medical School
Worcester, Massachusetts 01655

REPORT DATE: August 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2000	Annual (1 Aug 99 - 31 Jul 00)

4. TITLE AND SUBTITLE
99m Tc-peptides for Detection of Breast Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9280

6. AUTHOR(S)
Mary Rusckowski, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Massachusetts Medical School
Worcester, Massachusetts 01655

E-MAIL:
Mary.Rusckowski@umassmed.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*
The goal of this project is to identify peptides, using phage display peptide libraries, which bind with high affinity to the mutant EGFRvIII receptor, known to be in breast tumors. The peptides once selected, are radiolabeled with technetium-99m and tested for their potential as possible agents in the detection of breast cancer. Using a commercially available phage display peptide library we have identified two peptides which showed high consensus for cells expressing the mutant EGFRvIII receptor. Initial characterization of the selected peptides was by ELIZA and radiolabeled peptide cell binding studies. The labeled phage were also tested in mice with tumors. The group with tumors expressing the mutant receptor showed enhanced incorporation over the group with tumors expressing the wild-type EGF receptor. The consensus peptides were identified through sequencing the phage DNA . Both were synthesized , commercially, and then conjugated to the chelate NHS-MAG3 for radiolabeling with ^{99m}Tc. Both peptides have been tested in in vitro assays as above and tested in tumor bearing mice. Though the in vivo studies which are in progress, it is known that the ^{99m}Tc-peptide clears quickly, and there is an increase in accumulation with dose in the target.

14. SUBJECT TERMS
Breast Cancer, nuclear medicine, ^{99m}Tc-peptides, mutant epidermal growth factor, phage display libraries

15. NUMBER OF PAGES
19

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

The goal of this project is to select for a peptide, using phage display libraries, which recognizes an EGF mutant receptor present in tumor cells. Phage display is a methodology which offers great potential for cancer diagnostic agents. The peptide(s) selected will be radiolabeled with technetium-99m (^{99m}Tc) and tested *in vitro* and in *in vivo* mouse tumor models for their potential as agents for detection, through nuclear imaging, of breast cancers which expresses the mutant EGF receptor. If successful, this ^{99m}Tc-labeled mutant EGF-binding protein could serve not only as a useful agent in the diagnosis of breast cancer through imaging, but potentially be applicable to other cancers as well. Although not part of this proposal, the novel peptide selected as may be useful in therapy, by serving as a targeting agent in the delivery of therapeutic cancer drugs directly to cancer cells.

For these studies we are using phage display technology and the *in vitro* selection process called "biopaning" to screen commercially available phage display peptide libraries for a peptide(s) which recognizes the signature sequence of the mutant EGFRvIII and does not recognize the normal EGF receptor. Phage display describes a selection process for peptides or proteins which can bind to almost any target with affinities (K_d) that range from picomolar to micromolar. Thus, one is no longer limited to antibodies as binding proteins (Ladner, 1995). Phage Display peptides libraries contain random sequences of peptides of equal length, with a complexity of about 2×10^9 independent sequences.

BODY:

We have made significant progress in the first year on three of our four goals.

To review, the stated goals of this project are the following:

1. To select peptide(s) which binds to the mutant EGF receptor (EGFRvIII) with high affinity using Phage Display peptide libraries commercially available. Potentially four peptides (high affinity binders) are to be selected for further testing.
2. The selected peptides are to be conjugated to the NHS-MAG3 chelate and then radiolabeled with ^{99m}Tc for testing.
3. These high affinity EGFRvIII binding peptides once radiolabeled are to be tested *in vitro* and *in vivo*. Studies *in vitro* include maximizing labeling efficiency and specific activity, testing stability of peptides in serum, characterize binding to tumor cells in culture. Studies *in vivo* are to include the biodistribution and clearance properties in the normal mouse and in a mouse tumor model.
4. Lastly, the candidate peptides will be screened using surgically resected tumors from patients. If successful, a ^{99m}Tc-labeled mutant EGF-binding protein could serve as a useful agent in the diagnosis of breast cancer as well as other cancers which express the same mutant EGF receptor.

To date we have made substantial progress on points 1, 2, and 3, and progress within each area is described herein. To begin these proposed studies three appropriate cell lines were obtained from Albert J. Wong, and David K. Moscatella of Thomas Jefferson University, Phila, PA. One cell line, designated HC2 20d2/c, express the EGFRvIII, the mutant receptor, with about 2×10^6 receptors per cell. This cell line referred to by us in these studies as HC2 originated from NIH3T3 fibroblasts which were co-transfected with cDNA which corresponds to the 801 bp in

frame deletion. Cells to be used as controls and for subtraction steps during biopaning, were also obtained. One of these, the CO12 20c2/b expresses the normal EGF receptor with about 10^6 copies per cell. The other, the LTR b2, express the normal receptor but in a very low number of copies per cell, about 5-10 x 10^3 per cell. Fortunately for tumor models in nude mice, the HC2 cell line is tumorigenic. For a control tumor, the A431 cell line, currently used in this laboratory and which expresses the normal EGF receptor was used.

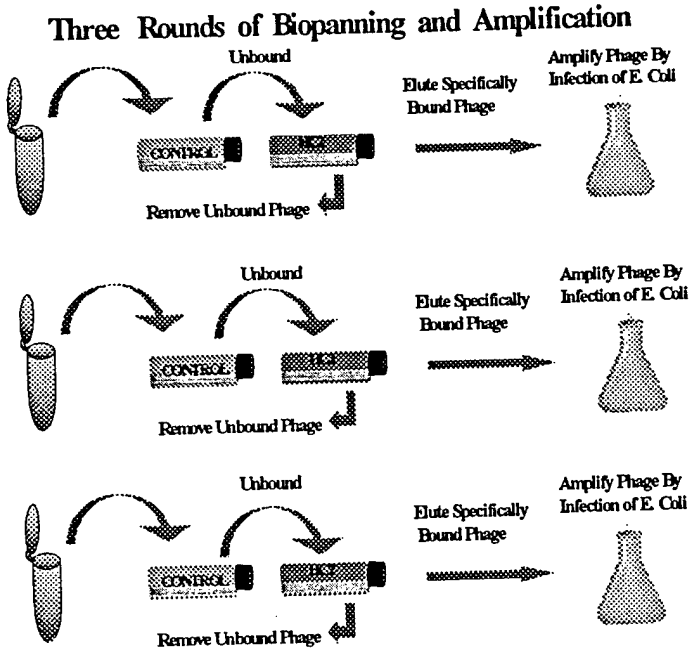
Biopanning

The peptide selection studies were begun with a phage display library purchased from New England BioLabs (Beverly, MA). The library contains randomized peptides of 12-amino acid residues, with a complexity of about 2 x 10^9 independent sequences. The DNA encoding for each peptide is fused to the gene coding for the end portion of the five coat filament proteins. The phage used in this kit is the filamentous M13 phage. With this unique peptide sequence on the end of the filament one can characterize the peptide ligands binding to cells.

The process of selection is outlined in the figure below. To briefly review the selection process, for these studies, biopaning is carried out by incubating the library of phage with a flask coated with the target cell. The unbound phage are removed by washing, and the bound phage are eluted by, for example, lowering the pH. The eluted pool is amplified, and the process repeated with an aliquot of the amplified phage. After repeating this process multiple times, individual phage clones are isolated and the DNA sequenced for identification of the unique peptide.

In our case, in the first round of selection a small volume of the phage pool (a 10µl volume contains approximately 100 copies of each of the 10^9 clones) was added to a flask containing the control cell, the LTR, as a subtractive step to pull out from the pool of phage any peptide which binds to other cell surface components. These cells are identical to the study cells, except they lack that one unique feature, the mutant EGF receptor. The phage which have not bound to the control cells were transferred to a flask containing the study cells, with the mutant receptor, the HC2's. After an incubation, the unbound phage were discarded, and the cell associated phage were eluted with a short incubation in 0.2M glycine pH 2. These eluted phage were amplified and then taken through three additional binding and amplification cycles to enrich the phage pool for those containing peptides with high binding for the HC2 cells. Throughout the selection process the time of incubation, elution conditions and temperature can be varied to select for peptides with various binding characteristics. In our case the incubation was kept to about 10 min at 37°C, and the peptides were eluted with 0.2M glycine buffer, pH 2.

After three rounds of selection and amplification, individual clones were isolated and the phage pool examined for consensus, a binding peptide.



The phage were surveyed through sequencing the DNA for the unique genetic site which codes for the filament peptide. To accomplish this a sample of the amplified phage were first grown on agar/agarose plates in a field of *E.coli*. The blue plaques which appear indicate a single phage clone. Isolated clones were removed, amplified, and the DNA isolated and prepared for sequencing. From the sequencing results of the selected plaques, the data was evaluated for consensus of amino acid residues.

Consensus sequences

Of the 20 plaques selected in our investigation, nine were identified with an identical amino acid sequence, and another set of four which shared a second common sequence. The seven remaining peptides showed some amino acids groups in common, but were not as complete as these two groups. So we went from a pool of 10^9 independent clones to a pool where nearly half were identical.

The consensus sequences are as follows:

Phage-3: H-Ser-**Pro-Trp**-Ser-**Glu**-Pro-Ala-Tyr-Thr-**Leu**-Ala-Pro-*Gly-Gly-Gly-Ser*-OH

Phage-5: H-Asn-Asn-**Pro-Trp**-Thr-**Glu**-Met-Arg-Ser-**Leu**-Leu-Ser-*Gly-Gly-Gly-Ser*-OH

Additionally, in the cell binding studies a third phage was used as a control: Phage-2.

Conjugation with MAG3 and ^{99m}Tc radiolabeling

To facilitate the evaluation process, the phage with the attached peptide were evaluated. In order to test the phage filament peptides for binding characteristics, a simple method to radiolabeled the

phage with ^{99m}Tc-was devised. Following the standard protocol used in our laboratory for conjugation of proteins and peptides with NHS-MAG3, the phage were conjugated to NHS-MAG3 in sodium bicarbonate buffer, pH 9. After a brief incubation, the conjugated phage can be separated from free MAG3, by a precipitation using polyethylene glycol. The phage pellet was then easily resolubalized in buffer.

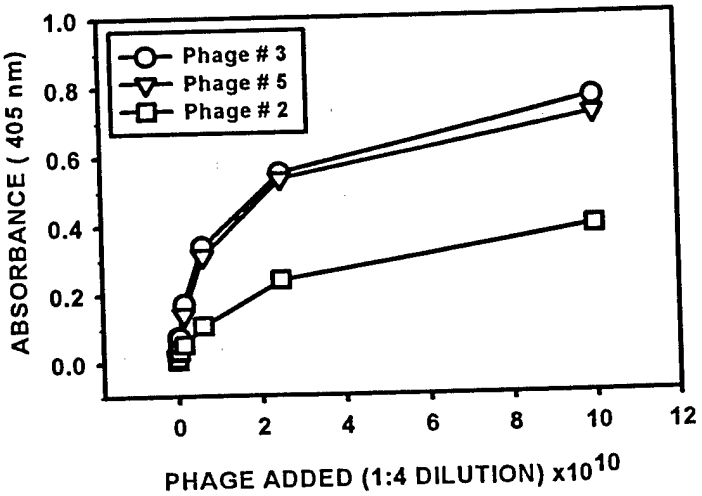
For radiolabeling with ^{99m}Tc, to 10^{11} phage in 0.1M PBS was added, ^{99m}Tc (pertechnetate), tin (1mg/ml in 10mM HCl) and tartrate . After a 45 min incubation, the labeled phage were removed from the solution again with precipitation with polyethylene glycol, set at 4°C for 30 min, and then spun to recover the labeled-phage pellet which was resolubalized in buffer.

The average radiolabeling efficiency of these phage was 82%, of which 18% was do to non-specific labeling. Typically, 160μCi was added to a pool of 10^{11} phage.

Cell binding assays

To test the selected peptide on the phage filaments a number of cell binding assays were performed. To begin, an ELISA assay was established to test the binding of the selected phage (and filament peptides) to the target cell, the HC2. In this assay the unlabeled phage preparations were tested against the study cell containing the mutant EGFvIII receptor, the HC2. Using a constant cell number and serial dilutions of the phage prep, we observed an increase in phage bound for all three phage preparations, which approached saturation in each case. As shown in **Figure 1**, Phage-3 and Phage-5 showed a similar level of binding, with Phage-3 showing somewhat higher binding than Phage-5. Phage-2 as a control phage, but still survived the selection process, showed a response which approached about half of that observed for Phage-3 and 5 in this particular test.

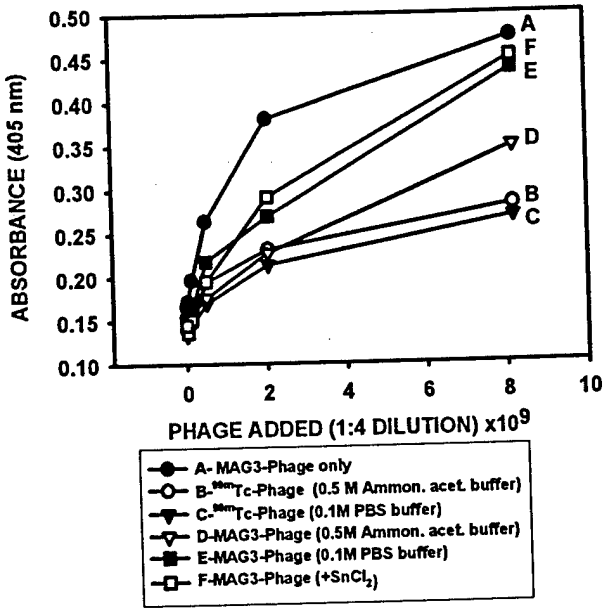
Figure 1.



Effect of radiolabeling on ELISA results

Since the phage preparations were taken through both the coupling procedure radiolabeling steps, there was concern that the coupling and labeling conditions may have had an effect on bioactivity. As shown in **Figure 2**, using an ELISA assay in assessing various buffers, solutions and manipulations, some loss in bioactivity was observed when taking the phage through a labeling procedure. Part of the loss may be due to the physical manipulation of the phage as well as loss due to sticking, and thus loss in phage titer as it is taken through various steps. There was no one agent which was alone responsible for the loss in bioactivity. Regardless of buffer or agent, after the labeling process, a loss in bioactivity was observed. In a measure of the titer of the sample a loss of about one order of magnitude was observed after radiolabeling, i.e., from 10^{11} to 10^{10} phage per unit volume.

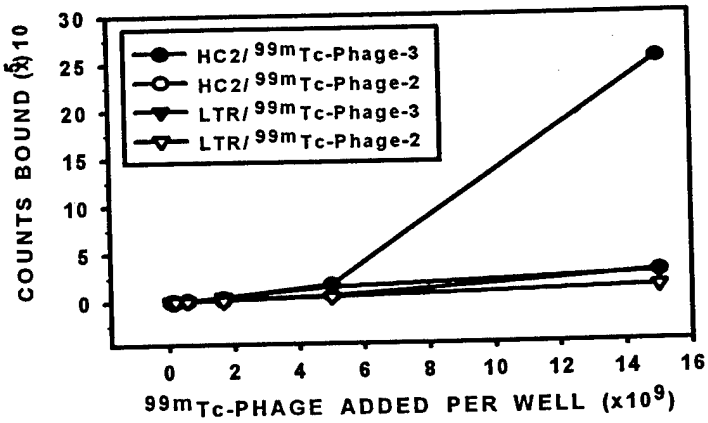
Figure 2.



Test of radiolabled Phage-3 and Phage-2 with HC2 and LTR (control) cells

The radiolabeled phage were tested for binding against the specific HC2 cells, and the control cells using a protocol similar to the ELISA. As illustrated in **Figure 3**, only the peptide showing a consensus sequence: Phage-3, shows an increase in binding (positive binding) to the study cells when compared to Phage-2, and to the control cell line.

Figure 3.



Competidtion Assay

Figures 4A and 4B illustrate a study using radiolabeled Phage-3, and Phage-5. In each case the activity bound is shown versus number of phage added using a constant cell number. Both the study cells and control cells are presented.

Figure 4A.

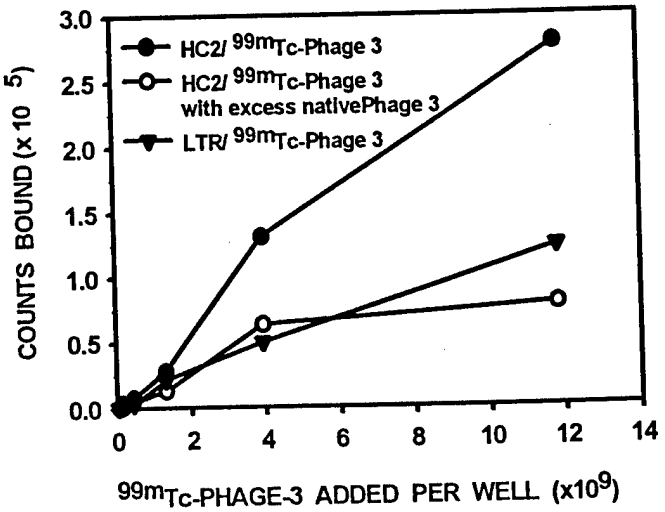
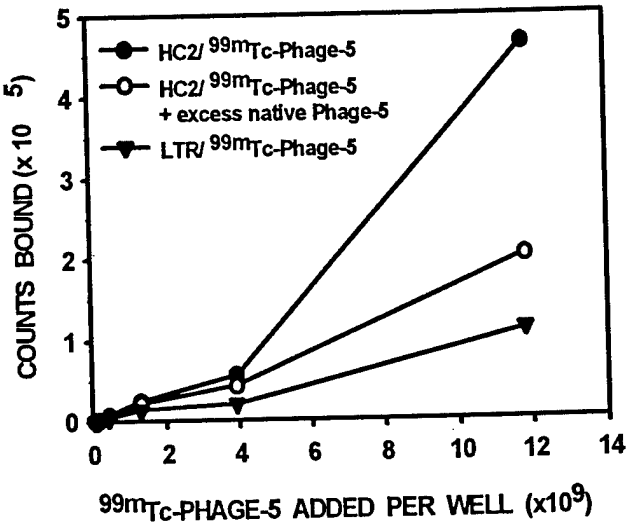


Figure 4B.

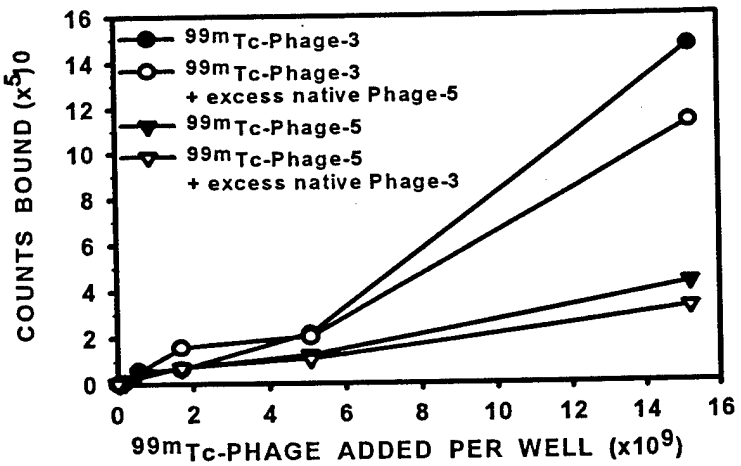


As in the ELISA an increase in phage bound was observed with phage added. To answer the question, is binding specific to a unique site? In both cases an excess of unlabeled phage (constant phage titer per well) was added to compete with the labeled phage. With the addition of excess unlabeled phage a reduction in labeled phage bound was observed. Thus suggesting that Phage-3 as well as Phage-5 peptides were competing for a specific site. Also shown in this study is the lower level of binding of the labeled phage to the control cells.

Cross competition test

In a similar study, the question addressed was, do phage-3 and phage 5 peptides shared the some binding site. As before, an excess of unlabeled phage was added, but this time to the radiolabeled phage-3 was added excess phage-5 (unlabeled). And vis-versa, to labeled phage-5 was added unlabeled phage-3. As shown in **Figure 5** there was some reduction in binding in both cases, but not to the level expected, so they may not share the same site, but the binding sites may be close enough to interfere with binding.

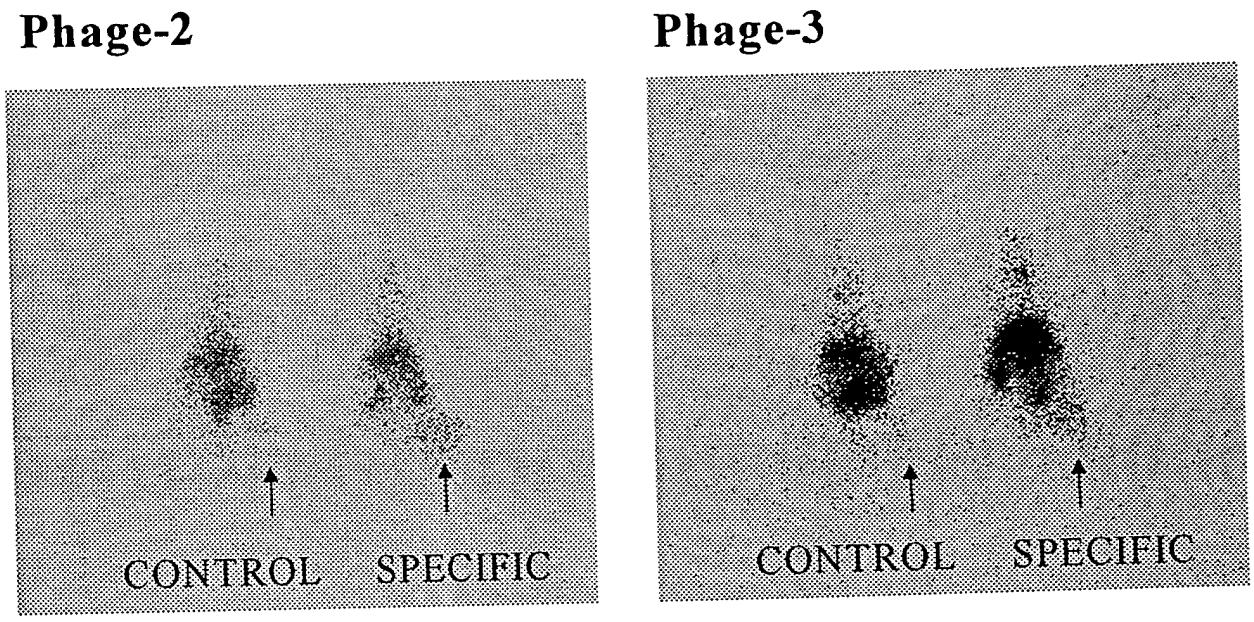
Figure 5.



Biodistribution in mice with tumors

The radiolabeled phage were also administered to mice with tumors in one thigh. Shown in the following figure (**Figure 6**.) are two sets of mice. One received the ^{99m}Tc-labeled phage-3 and the other ^{99m}Tc -labeled phage-2. The study included mice with the control tumor which expresses the normal EGF receptor. Minimal accumulation was seen in the control tumor, and both phage preparations show accumulation in the specific tumors. Which is shown on the right in both panels.

Figure 6.



There was also a remarkable difference in the biodistribution of the two phage. There was not only a difference seen in tissues between the two phage in animals with the same tumor type, but also for one phage type in mice with different tumors. For example, as illustrated in **Figure 7** here, for the phage-3 higher liver, spleen and blood activity in the study animal, than in another set of mice which have the control tumor. This was observed with both phage preparations. A difference in tumor localization was observed for the two phage as well. With the phage-2 showing greater accumulation in this particular case.

Figure 7A.

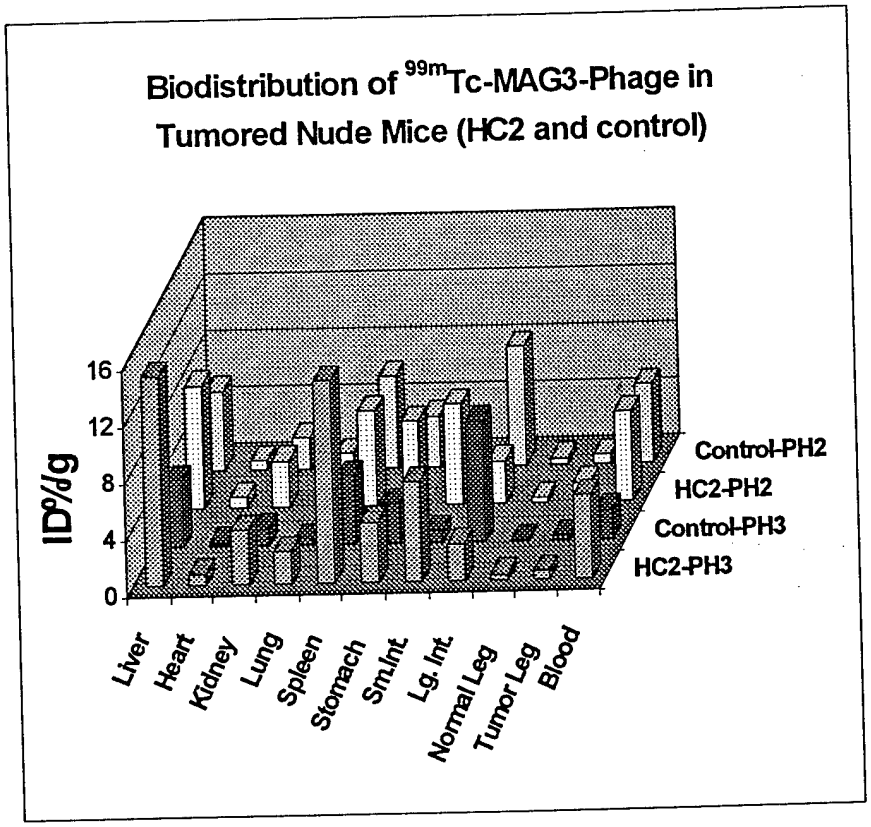
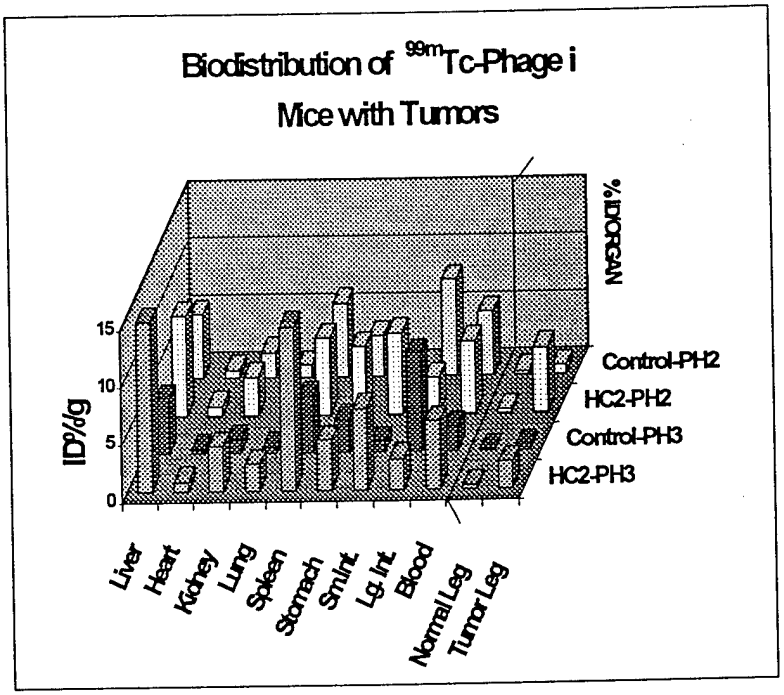
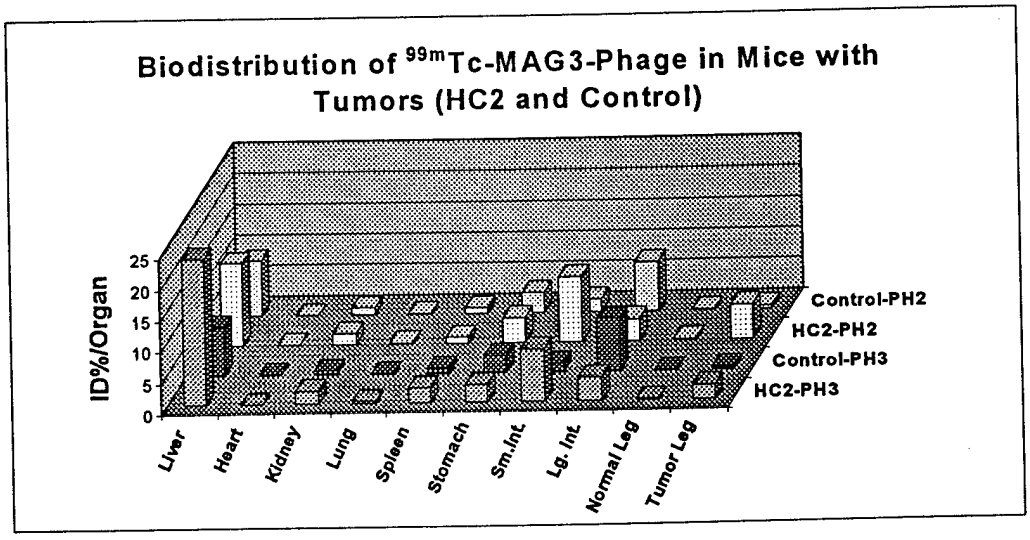

Biodistribution of 99mTc-MAG3-Phage in Tumored Nude Mice (HC2 and control)
ID%/g
16
12
8
4
0
Liver
Heart
Kidney
Lung
Spleen
Stomach
Sm.Int
Lg. Int
Normal Leg
Tumor Leg
Blood
Control-PH2
HC2-PH2
Control-PH3
HC2-PH3

Fig 7B and C.

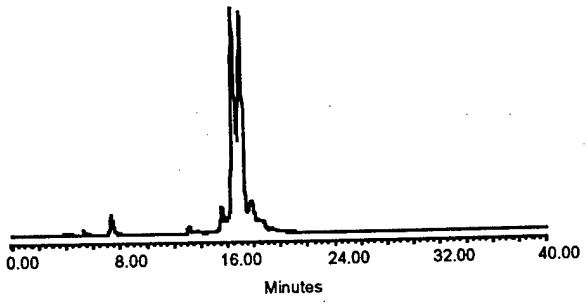

Biodistribution of 99mTc-Phage i
Mice with Tumors
%ID/ORGAN
ID%/g
15
10
5
0
Control-PH2
HC2-PH2
Control-PH3
HC2-PH3
Liver
Heart
Kidney
Lung
Spleen
Stomach
Sm.Int
Lg. Int
Blood
Normal Leg
Tumor Leg


Biodistribution of 99mTc-MAG3-Phage in Mice with Tumors (HC2 and Control)
ID%/Organ
25
20
15
10
5
0
Control-PH2
HC2-PH2
Control-PH3
HC2-PH3
Liver
Heart
Kidney
Lung
Spleen
Stomach
Sm.Int
Lg. Int
Normal Leg
Tumor Leg

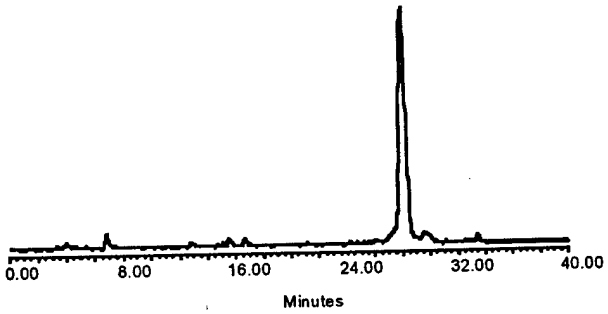
Synthesis of Peptides 3 and 5

The two peptides noted above and identified as Phage-3 and Phage-5 were synthesized commercially by Advanced ChemTech (Louisville, Kentucky) with a leader sequence of *GLY-GLY-GLY-SER-OH.* The peptides were conjugated to NHS-MAG3 in HEPES buffer pH 8.0 and peptide separated from free chelate on C18 Sep-PAK cartridge. A purification scheme was developed for both peptide to remove free chelate with varying the percentage of acetonitrile. After purification the peptide when radiolabeled had a radiochemical purity of greater than 90%. **Figure 8** shows the C18 profile of the radiolabeled peptides.

^{99m}Tc-MAG3-Peptide-3



^{99m}Tc-MAG3-Peptide-5



The labeled peptides were tested for binding to HC2 cells and challenged with an excess of unlabeled peptide. For these studies constant cell number (about 5 x 10^5) and serial dilutions of labeled peptide were incubated in eppendorf tubes. The labeled peptide ranged from 6 ug to 6ng per 10^5 cells.

To verify if binding was specific, a challenge with unlabeled peptide was included. With peptide-5 a decrease in binding was observed in the presence excess native peptide; but with peptide 3 the results were not as clear as shown in **Figure 9 A and B.**

Figure 9A.

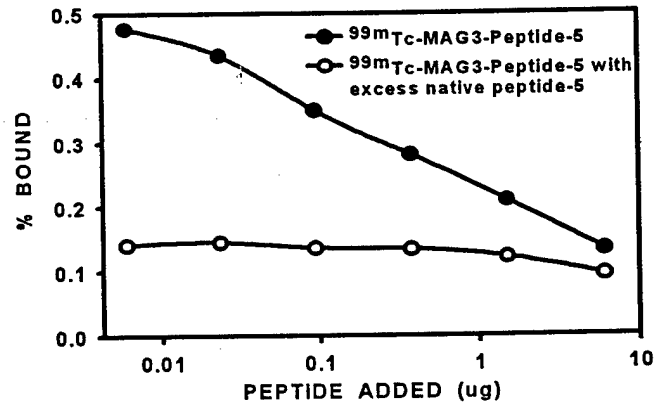
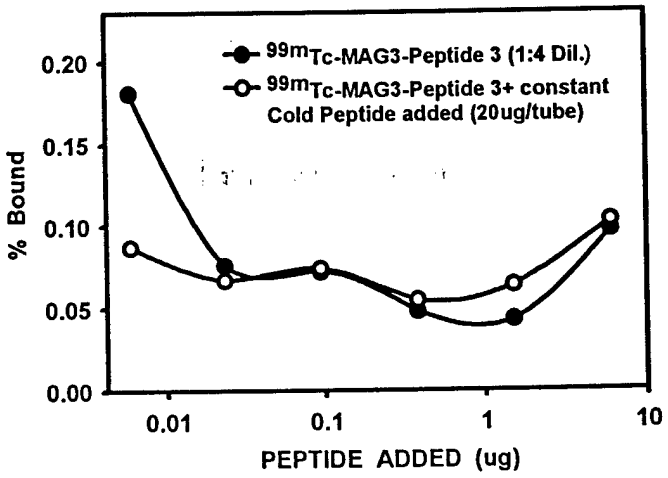


Figure 9B.

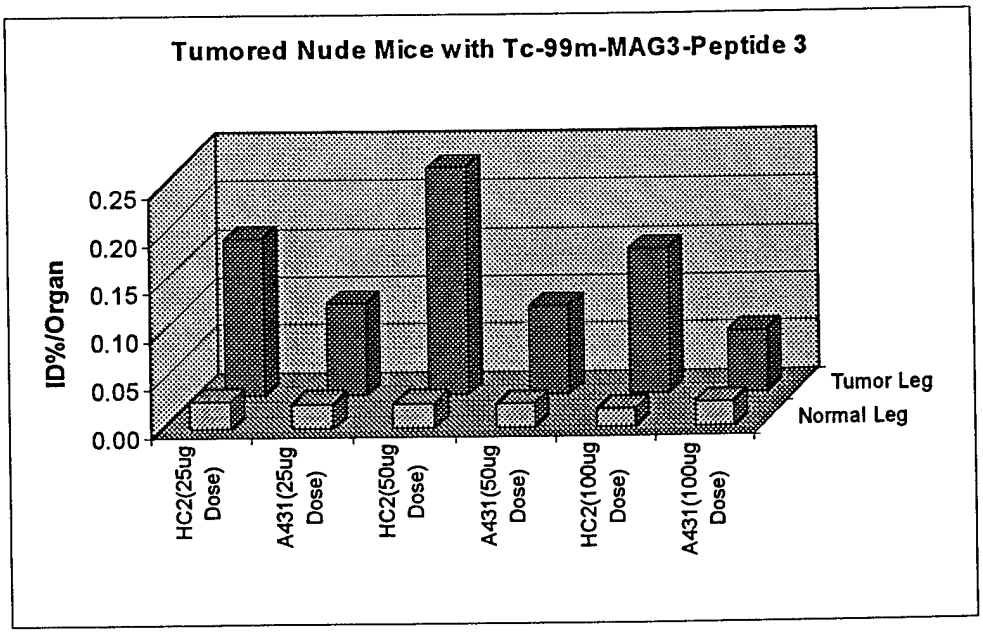


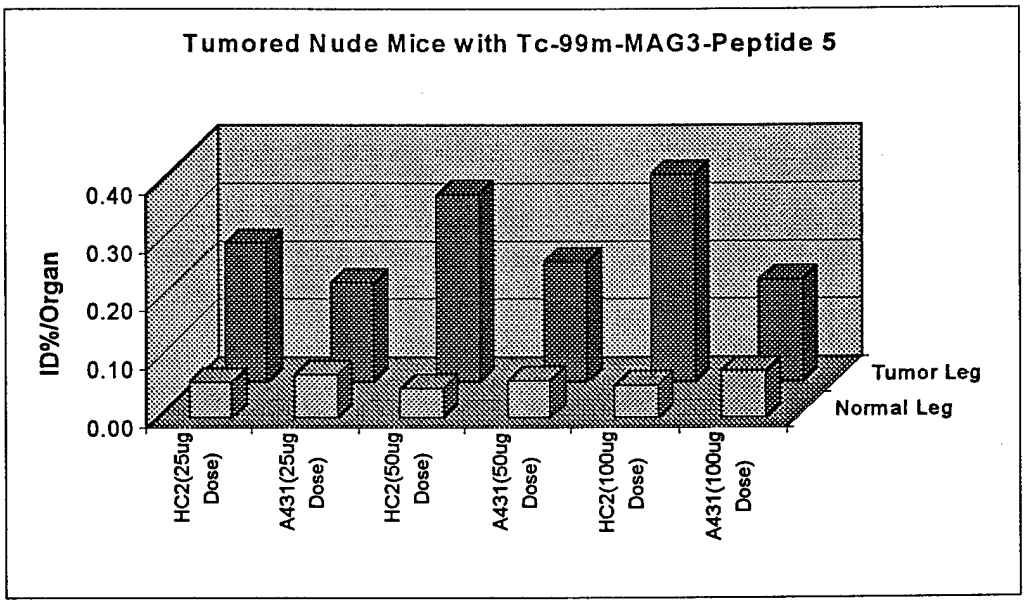
Labeled Peptides in Mice with Tumors

Both radiolabeled peptides were tested in nude mice with a tumor in one thigh. For the specific binding the tumor was HC2, and as control either the A431 or AHCN were used. First, a dose study with injection of 25μg, 50μg and 100μg, showed a better tumor to normal thigh ratio was observed at the highest dose. **(Figure 10)** Second, with a further test using 100 μg of peptide and the specific HC2 tumor and the ANCN. as control and sacrifice set at 18 hrs, although the target to normal tissue ratio were 1:6, the same was measured for the control mice as well. Third, to ascertain if binding could be specific in both tumor types, another set of mice were studied with a proadministration of unlabeled peptide, i.e., 1mg intraperitoneally at 30 min prior to the administration of labeled peptide intravenously. This study again showed no difference in tumor uptake in mice either with the specific or control tumor. Moreover, the high dose of native peptide did not reduce accumulation. Therefore it appeared that the first two candidate peptides were not successful in localizing in tumors in vivo mice.

The stability of the labeled peptides in serum incubated at 37°C showed a lower molecular weight species appearing after 24hrs, by size exclusion HPLC. The lack of successful targeting in mice may indicate that these two peptides are susceptible to protease digestion. Currently the selection process from the phage library is being repeated to select for another set of peptides.

Figure 10.




Tumored Nude Mice with Tc-99m-MAG3-Peptide 5
ID%/Organ
0.40
0.30
0.20
0.10
0.00
Tumor Leg
Normal Leg
HC2(25ug Dose)
A431(25ug Dose)
HC2(50ug Dose)
A431(50ug Dose)
HC2(100ug Dose)
A431(100ug Dose)

KEY RESEARCH ACCOMPLISHMENTS:

1. Using a phage display library, two consensus peptides were identified.

2. The phage corresponding to these two peptides were conjugated to NHS-MAG3 and radiolabeled with ^{99m}Tc.

3. The labeled phage peptides showed specificity of binding in cell binding studies.

4.The labeled phage peptides showed tumor accumulation.

5.The two consensus peptides were synthesized commercially, conjugated to NHS-MAG3 and radiolabeled with ^{99m}Tc.

6. The radiolabeled peptides show accumulation in tumors in mice but preliminary data suggests that the accumulation is not specific.

7. Selection studies continue for new peptides.

REPORTABLE OUTCOMES:

Abstracts

1. Society of Nuclear Medicine Annual Meeting June, 2000, St. Louis MO.
2. European Association of Nuclear Medicine, Paris, France, Sept 2-6, 2000.

CONCLUSIONS:

Two consensus peptides were identified in the first round of selection. Although the peptides anchored to the phage appeared promising in cell binding studies and in tumored mice, the pure peptides were not so encouraging. The lack of successful in mice may indicate that these two peptides may be susceptible to protease digestion. Also, it has been reported by others that the free peptide is not always as successful as the peptide tethered to the phage. The phage may serve to stabilize and position the peptide for binding. Currently the process is being repeated to select for another set of peptides. During the selection process there are many parameters to be examined, time of incubation, temperature and elution conditions. We began with one phage library and there are two other commercial libraries available, plus libraries from Dyax, Corp. Each of these will be examined.

REFERENCES

Ladner, RC. Constrained Peptide Binding Entities. In Phage Display of Peptides and Protein Domains: A Laboratory Manual, Kay B., Wiinter J., McCafferty, J. (Eds) Academic Press, 1995.